The Shared Behavioral and Biochemical Effects of Rapid-Acting Antidepressants Ketamine and Nitrous Oxide in a Chronic Corticosterone-Induced Animal Model of Depression

Master's Thesis

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While weeks of continuous treatment is required for conventional antidepressant drugs (e.g. fluoxetine) to bring their full therapeutic effects, a subanesthetic dose of ketamine alleviates the core symptoms of depression (anhedonia, depressed mood) and suicidal thinking within just few hours and the effects may last for days. Nitrous oxide (N_2O, "laughing gas"), another NMDAR antagonist, has recently been shown to have similar rapid antidepressant effects in treatment-resistant depressed patients (Nagele et al. 2015). We previously found using naïve mice ketamine and N_2O treatment to upregulate five mRNAs related to the MAPK pathway and synaptic plasticity, both implicated as being important in the action of rapid-acting antidepressants. In the current study, these shared mechanisms were further investigated in C57BL/6JHsd mice, using behavioral test batteries to study depressive-like behaviour and RT-qPCR for biochemical analyses. We first aimed to demonstrate behavioral differences between naïve mice and a chronic corticosterone-induced animal model of depression, and to use this model to investigate antidepressant-like effects of ketamine and N_2O. According to the results, chronic corticosterone produced some behaviors typical of a depressive-like phenotype, namely significant worsening of coat state and decreased saccharin consumption in the saccharin preference test. Both ketamine and N_2O exhibited antidepressant-like effects by reverting decreased saccharin preference. We then aimed to elucidate the effects of ketamine and N_2O on five previously found shared mRNAs: *Arc, Dusp1, Dusp5, Dusp6* and *Nr4a1*. N_2O significantly upregulated all targets in the vmPFC, except *Dusp5*, two hours after beginning of N_2O treatment. Neither ketamine nor sole chronic corticosterone produced any significant changes.

The results of this study suggest that N_2O is a potential candidate for rapid alleviation of depressive symptoms. We suggest that the action of rapid-acting antidepressants, more specifically N_2O, is based on a homeostatic response of the brain to a presented challenge. Here this challenge would be cortical excitation previously been shown to be caused by N_2O, which leads to activation of pathways such as MAPK and subsequent *Arc, Dusp* and *Nr4a1* signaling. The level of expression of these markers would then depend on which phase this response is in and hence, the differences in time between treatment and brain sample dissection could be a reason for conflicting results to previous research. Future studies would benefit from detailed investigation of the chronic corticosterone-induced model due to its potential in controlling for behavioral variability, thus reducing the number of animals needed for preclinical research. Overall the preliminary findings of this study could be one of the first steps in the search for the mechanisms underlying the potential antidepressant effect of N_2O, how these molecular markers are related to its action and how it differs from the action of ketamine.

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Ketamine, nitrous oxide, rapid-acting antidepressants, depression, animal model, corticosterone, MAPK, Arc, DUSP, Nr4a1

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Psykoterapia ja yleisesti käytössä olevat masennuslääkkeet vaikuttavat masennusoireisiin hitaasti, joten nopeavaikutteisimmille masennushoidoille on merkittävä tarve. Subanesteettinen annos ketamiinia pystyy lieventämään masennuksen oireita (anhedonia, masentunut mieliala) ja itsemurha-ajatuksia muutamien tuntien sisällä ja vaikutus voi kestää jopa muutaman päivän. Dityppioksidin (N_2O, "ilokaasu") on äskettäin osoitettu johtavan samankaltaiseen nopeaan masennuslääkevasteeseen (Nagele et al. 2015). Tutkimusryhmämme löysi sekä ketamiinin että ilokaasun lisäävän hiirissä viiden mRNA:n ekspressiota, jotka liittyvät MAPK-signalointiin ja synaptiseen muovautuvaisuuteen. Molemmat ovat tärkeitä nopeavaikutteisten masennuslääkkeiden toiminnalle. Tässä tutkimuksessa jatkettiin ketamiinin ja ilokaasun yhteisten taustamekanismien selvittämistä C57BL/6JHsd hiirissä, hyödyntäen käyttäytymiskokeita masennuksen kaltaisen käytöksen tarkasteluun ja RT-qPCR:ää biokemiallisia analyysejä varten. Ensiksi selvitimme kroonisen kortikosteronin juoton vaikutusta käyttäytymiseen, ja ketamiinin sekä ilokaasun kykyä muuttaa tätä. Krooninen kortikosteroni johti osittaiseen masennuksen kaltaiseen käyttäytymiseen, joka ilmeni turkin huonontumisella ja vähentyneellä sakkariiniliuoksen juomisella. Sekä ketamiini että ilokaasu palauttivat vähentyneen sakkariiniliuoksen juomisen kontrollien tasolle. Seuraavaksi lähdimme selvittämään ketamiinin sekä ilokaasun vaikutusta viiteen aikaisemmin löytämiimme yhteisiin mRNA-kohteisiin: *Arc, Dusp1, Dusp5, Dusp6* ja *Nr4a1.* Ilokaasu lisäsi merkittävästi kaikkien muiden paitsi *Dusp5* ekspressiota prefrontaalikorteksissa kaksi tuntia ilokaasun hoidon aloittamisen jälkeen. Kumpikaan ketamiini tai krooninen kortikosteroni itsessään eivät aiheuttaneet muutoksia.

Tämän tutkimuksen tulokset viittaavat siihen, että ilokaasu voi olla potentiaalinen ehdokas masennuksen oireiden nopealle lievitykselle. Tutkielmassa ehdotetaan nopeavaikutteisten masennuslääkkeiden – tarkemmin ilokaasun – vaikutusten johtuvan tietynlaisesta homeostaattisesta vasteesta haasteelle. Tässä kontekstissa haasteena toimii aivokuoren eksitaatio, jota ilokaasun on aiemmin havaittu aiheuttavan. Eksitaatio johtaa MAPK-reitin aktivaatioon ja seuraavaan *Arc-, Dusp-* ja *Nr4a1*-signalointiin. Tällöin mRNA-kohteiden tasot riippuisivat siitä missä faasissa homeostaattinen vaste on. Näin ollen aika hoidon ja aivonäytteiden dissektion välillä voi johtaa havaittuihin ristiriitoihin aikaisempien tutkimusten kanssa. Tulevaisuuden tutkimukset hyötyisivät krooniseen kortikosteronin juottoon perustuvan masennuksen hiirimallin yksityiskohtaisemmasta tarkastelusta, koska sillä voi olla mahdollisuus pienentää käyttäytymisen variaatiota ja näin ollen vähentää prekliiniseen tutkimukseen vaadittavia eläinmääriä. Tämän tutkielman tulokset havainnollistavat ilokaasun potentiaalisten nopeiden masennuslääkevaikutusten taustalla olevia tekijöitä, ja sitä kuinka nämä mahdollisesti eroavat ketamiinin toiminnasta.

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Abbreviations

ACTH	Adrenocorticotropin hormone
AMPAR	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor
ARC	Activity-regulated cytoskeleton-associated protein
BDNF	Brain-derived neurotrophic factor
CORT	Corticosterone
CRF	Corticotropin releasing factor
CUS	Chronic unpredictable stress
DUSP	Dual-specificity phosphatase
ECT	Electroconvulsive therapy
ERK	Extracellular signal-regulated kinase
GluR	Glutamate receptor
HPA axis	Hypothalamic-pituitary-adrenal axis
JNK	Jun N-terminal kinase
MAPK	Mitogen-activated protein kinase
MDD	Major depressive disorder
mTOR	Mammalian target of rapamycin
N_2O	Nitrous oxide (“laughing gas”)
NMDAR	N-methyl-d-aspartate receptor
NR4A1	Nuclear receptor subfamily 4 group A member 1
PFC	Prefrontal cortex
PVN	Paraventricular nucleus
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
SPT	Saccharin preference test
TrkB	Tropomyosin related kinase B
TST	Tail suspension test
ZT	Zeitgeber time

Review of literature

Introduction

Major depressive disorder (MDD) is a highly prevalent and debilitating psychiatric illness. Common symptoms include depressed mood, anhedonia, and cognitive dysfunction (American Psychiatric Association, 2013). The individual's emotional state is frequently dominated by negative thinking and low self-esteem, with recurring suicidal thoughts also being one of the hallmarks of the disease phenomenology (American Psychiatric Association, 2013). In addition to causing vast suffering on an individual level, MDD poses a large burden on society. Based on worldwide studies, depression has a lifetime prevalence of 10 % to 20 %, affecting currently more than 260 million people worldwide. According to The Global Burden of Diseases, Injuries, and Risk Factors Study it was the third most leading cause of years lived with disability in 2017, and its ranking is not decreasing (GBD Study 2017).

Depression and the hypothalamic-pituitary-adrenal axis

MDD is a highly complex disorder with high comorbidity rates and diverse etiologies, hence unravelling the mechanisms behind it is a challenge. It is generally thought that a combination of genetic predisposition, psychosocial factors and alterations in neurotransmission and hormone levels influence the development of depression (Nestler et al., 2002). Psychosocial factors such as exposure to stressful experiences are strongly associated with an increased risk for onset of depressive symptoms. This is especially the case with the most difficult treatment-resistant forms of depression (Kendler et al., 1999; Williams et al., 2016). The connection between stress and depression was initially drawn from observations of increased activity of the hypothalamic-pituitary-adrenal (HPA) axis, elevated cortisol levels and disrupted cortisol rhythm in depressed patients (Linkowski et al., 1987; Nestler et al., 2002).

The HPA axis is necessary for normal physiological and neurobiological responses to stress. One of its purposes is to prepare the body to cope with an acute stressor, and then to restore bodily homeostasis once the stressor has diminished. Exposure to an acute stressor activates the HPA axis resulting in a cascade of endocrine events (fig. 1). This cascade includes release and transport of corticotropin-releasing factor (CRF) from neurons in the paraventricular nucleus (PVN) of the hypothalamus to the anterior pituitary, where it stimulates the release of adrenocorticotropin hormone (ACTH) into the circulatory system. ACTH travels to the adrenal glands, where it stimulates the production and release of stress glucocorticoids (cortisol in humans, corticosterone in rodents) from the adrenal cortex into the circulatory

system. Glucocorticoids act on target tissues to regulate bodily functions and mobilize energy to deal with the stressor. Glucocorticoids also travel to multiple regions in the brain, most relevantly the PVN and hippocampus, where they exert an inhibitory influence in order to halt HPA axis activity through a negative feedback process. Although this is a normal process, prolonged activation of the HPA axis can present a serious health risk, leading to immunosuppression, growth inhibition, sleep disorders, impaired memory, decreased sexual behavior and chronic dysphoria (McEwen, 1998).

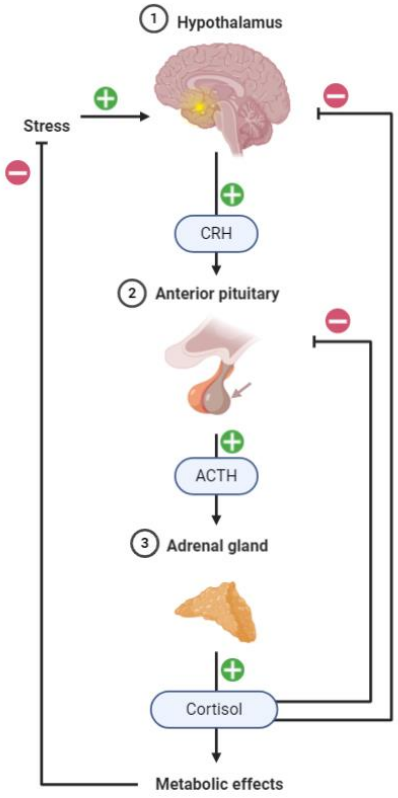

Figure 1.

Schematic representation of the hypothalamic-pituitary-adrenal (HPA) axis. Neurons in the paraventricular nucleus (PVN) of the hypothalamus synthesize corticotropin-releasing factor (CRF), which in response to stress is released and transported into the anterior pituitary gland. This induces release of adrenocorticotropic hormone (ACTH) into the systemic circulation. ACTH circulates to the adrenal cortex where it stimulates synthesis and secretion of glucocorticoids, such as cortisol (corticosterone in rodents). Glucocorticoids regulate various metabolic events and inhibit HPA axis activation in a negative feedback loop.

Elevated cortisol and the disrupted cortisol rhythmicity observed in depressed patients can be reversed by antidepressant treatment (Holsboer 2001; Linkowski et al. 1987). ACTH responses to exogenously administered CRF are also attenuated in depressed patients, with the relationship being most clear in depressed patients with the most severe hypercortisolism (Gold et al., 1986). Furthermore, patients with Cushing's disease, a disorder marked by chronically high levels of cortisol, show high rates of depression (Gold et al., 1986). These findings provide a strong basis for the possibility that some aspects of depression may arise from a dysregulated HPA axis.

Rapid-acting antidepressant ketamine

The complexity of MDD is reflected in the inefficacy of current treatments. Depending on the treatment, only about 20 % to 50 % of patients demonstrate complete remission while up to

80 % show partial responses (Nestler et al., 2002). These treatments are also sub-optimal in another significant manner: time. For those patients who do respond to current treatments, either antidepressant drugs or psychotherapy, the therapeutic effects become evident with a delay of several weeks or even months (American Psychiatric Association, 2013; Nestler et al., 2002).Taking into consideration the association between MDD and high rates of suicide, this lag in therapeutic effect can be fatal. Electroconvulsive therapy (ECT) can provide more rapid and significant improvements in depressive behavior and it has traditionally been the most effective treatment form in treatment-resistant patients (Kodama et al., 2005). However, the use of ECT is limited by available resources and cognitive adverse effects. The interest for developing novel rapid-acting antidepressants reached new heights when Berman et al. (2000) discovered that a low-dose ketamine infusion leads to robust decreases in depressive symptoms already within one-to-three days in patients diagnosed with MDD.

The neurobiological mechanisms underlying the rapid antidepressant effects of ketamine have not yet been fully elucidated. The current consensus is, however, that rapid facilitation of glutamatergic neurotransmission and subsequent activation of brain-derived neurotrophic factor (BDNF) receptor tropomyosin related kinase B (TrkB) signaling in limbic brain structures and the consecutive increase in synaptic plasticity and synaptogenesis are important (Zanos et al., 2018). Notably, the behavioral effects of ketamine sustain even after the drug has been eliminated from the brain (Clements & Nimmo, 1981; Zanos et al., 2018), indicating that the underlying increases in synaptic plasticity and synaptogenesis are not only induced rapidly but are able to maintain persistent changes in behavior.

Numerous mechanisms have been proposed to underlie ketamine's rapid facilitation of glutamatergic neurotransmission. The most popular suggestion is that subanesthetic ketamine selectively blocks N-methyl-d-aspartate receptors (NMDARs) expressed on gamma aminobutyric acid inhibitory interneurons, which leads to lack of inhibitory feedback and a successive surge in glutamate and excitatory drive in the prefrontal cortex (PFC) (Autry et al., 2011; Li et al., 2010; Zanos et al., 2018). This leads to indirect modulation of postsynaptic glutamatergic α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs), which are integral to synaptic plasticity (Koike et al., 2011; Li et al., 2010; Maeng et al., 2008; Zhou et al., 2014). Additionally the further activation of BDNF receptor TrkB, mammalian target of rapamycin (mTOR) and mitogen-activated protein kinase (MAPK) signaling along with the inhibition of GSK3β have all been associated with ketamine's' antidepressant effects (Beurel et al., 2011; Lepack et al., 2014; Li et al., 2010; Réus et al., 2014). Pre- and co-

treatment with a selective AMPA receptor inhibitor, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[f]quinoxaline-2,3-dione (NBQX), has been shown to block the induction of these different signaling pathways and antidepressant-like behavioral effects in rodents (Autry et al., 2011; Li et al., 2010; Zhou et al., 2014).

Activation of mTOR has been functionally linked with local protein synthesis in synapses, resulting in the production of proteins required for the formation, maturation, and function of new spine synapses (Li et al., 2010). Ketamine administration has been shown to transiently increase phosphorylation of mTOR, which led to sustained increase in the levels of synapse-associated proteins (Li et al., 2010). This increase and the antidepressant actions of ketamine could be blocked with an mTOR inhibitor rapamycin, indicating that ketamine induced synaptogenesis and subsequent therapeutic effects occur mainly through mTOR signaling (Li et al., 2010). In contrast to the actions of NMDA antagonists, such as ketamine, acute or chronic administration of conventional monoaminergic antidepressants are proposed not to increase mTOR signalling (Li et al., 2010; Zanos et al., 2018), although this has not been thoroughly investigated.

Nitrous oxide

Nitrous oxide (N_2O, "laughing gas") is a widely used anesthetic and analgesic. Nagele et al. (2015) investigated N_2O in treatment of MDD largely because its mechanism of action is based on inhibiting NMDA receptors like ketamine. In their Proof-of-Concept trial, 20 patients with treatment-resistant depression were randomly assigned to either 1-hour inhalation of 50% nitrous oxide/50% oxygen or 50% nitrogen/50% oxygen. Compared with placebo, N_2O significantly reduced depressive symptoms at 2 hours and 24 hours after the treatment. Although the primary target of N_2O is the NMDA receptor, it acts on other receptors as well. It can cause stimulatory activity at dopaminergic, $\alpha 1-$ and $\alpha 2-$ adrenergic and opioid receptors (Banks & Hardman, 2005). Especially interaction with the opioid system is proposed to be the main mechanism behind its analgesic effects. N_2O does not undergo biotransformation (in humans or mice) and its low solubility allows it to be eliminated from the body rapidly almost entirely via the lungs (Banks & Hardman, 2005). This extremely fast pharmacokinetic profile of N_2O provides a unique opportunity to study the timeframe of the neurobiological mechanisms behind the action of rapid-acting antidepressants.

The MAPK pathway, depression and rapid-acting antidepressants

One important signaling pathway shown to be necessary for not only ketamine-induced mTOR activation but also successive antidepressant effects is the MAPK pathway (Gourley et al., 2008; Li et al., 2010; Réus et al., 2014). This cascade consists of types of protein kinases that are specific to the amino acids serine (Ser, S) and threonine (Thr, T). The MAPK family has three identified subfamilies, Jun N-terminal kinase (JNK), extracellular signal-regulated kinases (ERKs, also known as MAPK1/3) and p38MAPK (Pérez-Sen et al., 2019; Réus et al., 2014). MAPK1/3 primarily regulate cellular growth and differentiation, whereas p38MAPK and JNK mainly function as mediators of cellular stress (Meller et al., 2003). The subfamily most commonly associated with the actions of rapid-acting antidepressants is the MAPK1/3 family. Although, studies have suggested all three MAPK subfamilies to modulate AMPAR potentiation produced by the antidepressant tianeptine (Szegedi et al., 2011), suggesting a more extensive association between the glutamatergic system and the whole MAPK pathway. Shirayama et al., (2002) have shown that antidepressant effects produced by infusion of BDNF into the hippocampus are blocked by inhibiting the function of MAPK kinase, a MAPK1/3 activator. Prevention of phosphorylation of MAPK1/3 and antidepressant effects produced by several antidepressants has also been demonstrated with a similar inhibitor, PD184161, in several animal models of depression (Duman et al., 2007; Réus et al., 2014).

Reduced MAPK pathway activity itself has been associated with depressive behavior. It has been shown to be downregulated in the cortex and hippocampus of depressed humans and in various animal models of depression (Duric et al., 2010; Dwivedi et al., 2001; Meller et al., 2003). Both MAPK1/3 transcription and translation, and its kinase activity were reduced in the PFC and hippocampus in post-mortem tissues of depressed patients and suicide victims compared to controls (Duric et al., 2010; Dwivedi et al., 2001). Similarly, upstream activators of the MAPK pathway, such as Raf kinases and MAPK kinase, were downregulated in their kinase activity (Duric et al., 2010). Furthermore, acute injection of PD184161 itself produces depressive-like effects in preclinical behavioral tests used to model depression, learned helplessness (LH), forced swimming test (FST) and tail suspension test (TST), although for TST the effect was only seen during the first three minutes not during the total six minutes of testing (Duman et al., 2007). These findings indicate that reduced MAPK signaling leads to depressive-like behavior and that activation of this signaling pathway by rapid-acting antidepressants such as ketamine may play a role in reversing this behavior.

In contrast to chronic stress, which is usually associated with MDD, preclinical studies have demonstrated that acute stress affects MAPK pathway activation differently. Acute restraint stress increased phosphorylation of MAPK1/3 in the hippocampus and PFC (including the cingulate cortex), whereas phosphorylation and expression of MAPK1/3 was downregulated in various chronic stress models of depression (Meller et al., 2003). However, the increase caused by acute stress was only transient, with phosphorylation levels returning to baseline 30 minutes after termination of stress (Meller et al., 2003). Acute stress did not produce any effect on phosphorylated JNK and p38MAPK levels (Meller et al., 2003), whereas repeated restraint stress led to reduced phosphorylation of all three MAPKs although only in the PFC (Meller et al., 2003). The same phenomenon has been reproduced with other types of manipulations which are considered as forms of acute stress, such as ischemia and electroconvulsive shock (preclinical form of ECT; Meller et al., 2003). These results suggest there to be an initial widespread activation of MAPK signaling upon exposure to acute stress, which then diminishes after the stressor is discontinued.

An important aspect of MAPK pathway function is its regulation by dephosphorylation. The specific dephosphorylation of MAPKs on both threonine and tyrosine residues is mediated by a subfamily of cysteine-dependent dual-specificity protein phosphatases (DUSPs) also known as MAPK phosphatases (MKPs) (Pérez-Sen et al., 2019). DUSPs can control MAPK signaling very robustly, since they are able to deactivate MAPKs with a 10-100 times stronger potency than the upstream kinases (Pérez-Sen et al., 2019). Each member of the DUSP family shows distinct substrate specificities for MAPKs, specific tissue distributions and subcellular localizations, and different modes of expression inducibility by extracellular stimuli (Pérez-Sen et al., 2019). Initially mitogens activate MAPKs, which in turn promotes DUSP expression (Pérez-Sen et al., 2019). Traditionally, DUSPs have been a major focus of cancer research due to their well-documented participation in the regulation of cell proliferation. However, it has been shown that expression of DUSPs is upregulated in depressed patients and in animal models of depression, and in preclinical studies several antidepressants reverse the inhibition of MAPKs observed in these same animals (Duric et al., 2010; Gourley, Wu, & Taylor, 2008).

Our research group has performed a study in which we observed shared effects on mRNA expression between subanesthetic ketamine and N_2O treatments in naïve mice using mRNA sequencing. Some of the most significant effects shared by both treatments were seen in markers that play important roles in MAPK signaling (Rozov et al., unpublished data).

Certain *Dusp*s were among the most affected. Interestingly, their expression was upregulated, just as has been shown with depressed patients and animal models of depression. The three most significantly upregulated *Dusp*s were *Dusp1*, *Dusp5* and *Dusp6*. In addition to these, ketamine and N_2O upregulated *Arc* and *Nr4a1*, which can affect synaptic organization. Few studies have focused on how these five gene markers are affected by rapid-acting antidepressants in animal models of depression. Hence, they were chosen to be analyzed in my thesis. Next, I will give a brief introduction on these target genes and their association to MDD and rapid-acting antidepressants.

Dusp1

Depending on the cellular model and context, DUSP1 inactivates MAPK1/3, p38MAPK and JNK. A whole-genome expression analysis study on post-mortem hippocampal tissues showed significantly increased expression of *Dusp1* in the hippocampal subfields of subjects with MDD compared to matched controls (Duric et al., 2010). In addition, increased hippocampal *Dusp1* expression as a result of viral-mediated gene transfer in mice and rats causes depressive-like behaviors, and expression is also increased from chronic unpredictable stress (CUS). Fluoxetine (a serotonin-selective reuptake inhibitor, an antidepressant) attenuated this increase (Duric et al., 2010). Sustained induction of *Dusp1* would lead to inhibition of MAPK signaling, and this inhibition has been previously demonstrated in the hippocampal tissue of depressed individuals who committed suicide (Dwivedi et al., 2001). Gourley et al. (2008b) showed that antidepressants reversed inhibition of MAPK1/3 phosphorylation in the dentate gyrus when they produced anti-depressant effects on behavior. Studies demonstrating the effects of rapid-acting antidepressants on *Dusp1* expression itself provide conflicting results. A microarray analysis study found that *Dusp1* was downregulated in the anterior cingulate cortex of naïve mice treated with a single subanesthetic dose of ketamine (Orozco-Solis et al., 2017). However, electroconvulsive shock was shown to upregulate *Dusp1* levels 1 hour after administration in the rat PFC (Kodama et al., 2005).

Dusp1 is also a stress- and glucocorticoid-responsive immediate-early gene, which supports the role of stress in depression. Duric et al. (2010) studied the relationship between *Dusp1* and stress by utilizing conventional *Dusp1*-/- mice. These mice did not differ from wild type mice under baseline circumstances. However, exposure to CUS did not cause a reduction in sucrose consumption in these mice whereas with wild type mice a progressive, significant reduction was observed. This suggests that the behavior of *Dusp1*-/- mice are comparable to control in the absence of stress and are resistant to CUS-induced anhedonia. Exposure to

CUS decreased the amount of phosphorylation of both MAPK1/3 in wild type mice (Duric et al., 2010). In contrast there was no significant effect of CUS on MAPK1/3 phosphorylation levels in *Dusp1*-/- mice compared to non-stressed controls, and there were no significant effects on total MAPK1/3 levels under any of the conditions tested. These results indicate that *Dusp1* is required for decreased activation of MAPK signaling, as well as depressive behavior such as anhedonia, in response to CUS.

Dusp1 expression is also controlled by BDNF signaling. Three hours of treatment with BDNF induced DUSP1 protein expression at different developmental stages *in vitro* (Jeanneteau et al., 2010). *Dusp1* mRNA is downregulated in the PFC of conditional forebrain *bdnf*-/- mice and BDNF-dependent induction of *Dusp1* was blocked by pre-incubation with a non-selective tyrosine kinase inhibitor K252a. *Dusp1* was also induced during neuronal excitation caused by KCl, and this response was decreased by the same inhibitor, suggesting that neuronal excitation leading to endogenous BDNF release and following TrkB signaling is needed for *Dusp1* induction (Jeanneteau et al., 2010). As for the effects of *Dusp1* signaling on synaptic plasticity, it has been shown to control the complexity of axonal branching *in vivo* and *in vitro* (Jeanneteau et al., 2010). The outgrowth of axons from cultured cortical neurons decreased upon prolonged *Dusp1* overexpression from days *in vitro* (DIV) 1-3, which occurred concomitantly with the dephosphorylation of MAPK1/3, p38MAPK and JNK. Later, JNK was shown to be the main neuronal MAPK substrate of *Dusp1* that controls axonal shape. The dephosphorylation of JNK led to a decrease in microtubule stability seen as different microtubule post-translation modifications.

Dusp5

Dusp5 is an inducible gene for a dual-specificity phosphatase, which specifically interacts with and inactivates MAPK1/3 in mammalian cells (Kucharska et al., 2009). Induction of *Dusp5* mRNA and protein in response to growth factors is also dependent on MAPK1/3 activation and the accumulation of DUSP5 protein is tightly regulated by rapid degradation (Kucharska et al., 2009). DUSP5 is phosphorylated by MAPK1/3 both *in vitro* and *in vivo* on three sites (Thr321, Ser346 and Ser376) within its C-terminal domain (Kucharska et al., 2009). This interaction with MAPK1/3 and transient nature of the protein suggests that *Dusp5* functions as a part of a negative feedback mechanism to specifically control the duration and magnitude of MAPK activation. A study has shown *Dusp5* to be downregulated by a single dose of ketamine in the anterior cingulate cortex of naïve mice (Orozco-Solis et al., 2017).

Otherwise *Dusp5* has not been, to my knowledge, previously studied in the context of rapid-acting antidepressants.

Dusp6

Dusp6 codes for a MAPK-specific phosphatase that can be slowly induced and is phosphorylated upon activation of the mTOR pathway (Bermudez et al., 2008; Pérez-Sen et al., 2019). This phosphorylation is interesting, since it not only activates the protein but also reduces the half-life of the phosphatase as well (Bermudez et al., 2008), indicating that it also has a transient nature. As with *Dusp5,* very little is known of the role of *Dusp6* in MDD and rapid-acting antidepressants. In the same study by Orozco-Solis et al. (2017), *Dusp6* expression was also reduced by ketamine treatment. In contrast to this, Kodama et al. (2005) showed that *Dusp6,* like *Dusp1*, was upregulated in the rat PFC after electroconvulsive shock.

Arc

Activity-regulated cytoskeleton-associated protein (Arc) is considered a plasticity protein, which is coded by a member of the immediate-early gene family, a rapidly activated class of genes (Link et al., 1995). Synaptic activity in an NMDA receptor-dependent manner increases *Arc* mRNA expression in the rat cerebral cortex 5 minutes after stimulation and this may last for over 8 hours (Rial Verde et al., 2006). After induction, *Arc* mRNA is rapidly trafficked into dendrites, where it and its protein localize in active synapses (Link et al., 1995; Rao et al., 2006; Steward & Worley, 2001). The precise function of *Arc* at synaptic sites in unknown, but some studies show it regulates AMPA receptor trafficking (Rao et al., 2006; Rial Verde et al., 2006). Increased *Arc* expression in hippocampal CA1 pyramidal neurons reduced the amplitude of currents mediated by AMPARs, and this reduction is prevented by RNAi knockdown of *Arc* (Rial Verde et al., 2006). The reduction of AMPAR mediated currents happens through endocytosis of AMPARs containing GluR2 but not GluR1 subunits (Rial Verde et al., 2006). This regulation seems to be bidirectional, since inhibition of AMPA receptors strongly potentiated activity-dependent *Arc* transcription (Rao et al., 2006). Another important factor regulating *Arc* transcription is the MAPK pathway. *Arc* transcription has been shown to be dependent upon activation of the MAPK cascade (Waltereit et al., 2001). Furthermore, BDNF strongly elevates *Arc* mRNA levels and upregulates Arc protein levels (Rao et al., 2006), and this effect is blocked by two inhibitors of the MAPK pathway, U0126 and PD98059 (Rao et al., 2006). *Arc* has been shown to be transiently upregulated by ketamine both *in vitro* and *in vivo* (Choi et al., 2015; Kohtala et al., 2019; Li et al., 2010), and by acute stress (Molteni et al., 2010). However, it has seldom been studied in relation to

chronic stress or other animal models of depression. Chronic mild stress caused reduction in *Arc* in the PFC and CA1 region of the hippocampus, and this reduction was present also in a *VGLUT*+/- animal model that showed depressive behavior (Elizalde et al., 2010).

Nr4a1

The nerve growth factor IB (NR4A1, nuclear receptor subfamily 4 group A member 1; also known as Nur77) is a protein that is encoded by the gene *Nr4a1*. It is a key regulator of macrophage gene expression during inflammation as shown with treatment with lipopolysaccharides (Pei et al., 2006). Although this is the context *Nr4a1* has largely been studied in, it has been shown to act as an activity-dependent immediate early gene responding to a variety of stressors and sensory stimuli. *Nr4a1* transcription-induced signaling is able to change the expression of target genes involved in mitochondrial functions and activate the AMP-activated protein kinase catabolic pathway (Jeanneteau et al., 2018). Problems in this processing pathway due to prolonged stress could lead to metabolic impairments such as excessive oxidative stress in response to environmental stimuli. Chronic stress and corticosterone increased activity on the transcriptional level of neuronal *Nr4a1* in a mouse cortex compared with controls at rest (Jeanneteau et al., 2018). *Nr4a1* was also upregulated in the hippocampus of patients with MDD in the same whole-genome microarray profiling study mentioned in association with *Dusp*s (Duric et al., 2010). Additionally, qPCR showed higher *Nr4a1* mRNA levels in the medial PFC of depressed patients compared to healthy subjects (Jeanneteau et al., 2018). *Nr4a1* could relate to depressive behavior by affecting synaptic organization. Indeed, it has been shown to cause dendritic spine deterioration *in vivo* in the PFC and silencing endogenous *Nr4a1* expression protected against stress- and corticosterone-mediated dendritic spine loss. This silencing also reduced immobility in TST (Jeanneteau et al., 2018), indicating that *Nr4a1* function does have an effect on behavior.

Finally, the genes encoding *Nr4a* family transcription factors have been shown to be regulated by signaling mechanisms critical for memory formation (Hawk & Abel, 2011). These genes also show increased expression levels after learning in multiple brain structures (Hawk & Abel, 2011). This along with the results on dendritic spine deterioration implies that *Nr4a1* might be related to the mechanisms of rapid-acting antidepressants through synaptic plasticity regulation. An *in vitro* study showed that ketamine upregulated expression of *Nr4a1* (Choi et al., 2015). Due to the little amount of research available, further investigation is needed to further elucidate the role of *Nr4a1* and the other molecular markers in not only MAPK signaling but also the action of rapid-acting antidepressants.

Animal model of depression based on chronic exposure to corticosterone

Studies focusing on rapid-acting antidepressants are often only performed with naïve animals. While these studies offer valuable insights, it is still not known for certain whether the neurobiological effects of rapid-acting antidepressants in naïve animals differ from those in animal models of depression. Many different types of rodent models of depression have been used and validated extensively, such as CUS or social defeat stress. A majority of these are based on introducing a stressor to the mice or rat for a prolonged period of time, which mirrors the major risk factors for development of MDD, and the behavioral consequences are ameliorated by antidepressants. Still, studying depression in rodents can be a difficult task. Not only are depressive syndromes in humans heterogeneous and their etiologies diverse, but important symptoms such as guilt and suicidality are impossible to reproduce in animal models. Another problem is the variability in behavior exhibited by animal models. For instance, some studies suggest repeated restraint stress and chronic mild stress produces changes in anxiety, depressive behavior and aggression (Duric et al., 2010; Meller et al., 2003) whereas others show no effect (Gregus et al., 2005). A possible cause for this variation is that stressful stimuli can differ in terms of their physical and psychological qualities. They are also quite sensitive to procedural differences between experiments, which may result in differing circulating corticosterone levels between rodents exposed to the same stressor. In addition, many animals habituate to aversive effects of these stressors when they are repeatedly exposed to them (Gregus et al., 2005). When the goal is to produce more robust and reliable behavioral symptomatology, it is desirable to avoid this kind of variation. While no physical or behavioral stress-induced depression-like syndrome in rodents can fully recapitulate the human condition, a model that produces a persistent and extensive endophenotype sensitive to antidepressant treatments would be a valuable tool for understanding the neurobiological responses to rapid-acting antidepressants.

An animal model of depression has been suggested whereby exogenous corticosterone is administered over a period of weeks to months by subcutaneous injection, pellet implantation, osmotic pump infusion, or administration via drinking water (Gourley, et al., 2008a). Corticosterone, as discussed previously, is a critical mediator of stress effects on behavior, and chronic corticosterone administration recapitulates important aspects of the neuroendocrine response to prolonged stress. These animals have shown anhedonia in sucrose/saccharin preference tests, decreased sexual behavior, immobility in forced swim and tail suspension test, and decreased grooming behavior - all measures typically used to infer a

depressive phenotype (Gourley, et al., 2008a; Gourley, et al., 2008b; Moda-Sava et al., 2019). Many of the changes associated with repeated corticosterone administration can be reversed with antidepressant treatment (Gourley, et al., 2008b; Moda-Sava et al., 2019). In addition to affecting behavior, glucocorticoid exposure leads to molecular and anatomical level changes in the brain also seen in patients with depression. Especially the hippocampus and PFC express high amounts of glucocorticoid receptors, and elevated corticosterone levels have been suggested to lead to reduction of synapses and thereby hippocampal volume, and impairments in BDNF mediated signaling in the PFC (Gourley, et al., 2008a; Holsboer, 2001; McEwen, 1998).

This model was chosen for my thesis because of its direct connection to the HPA axis, a factor consistently associated with MDD. The goal in using this model was to explore the use of a more straightforward approach for modeling depression thus possibly lessening behavioral variability and the need for large cohorts of animals in preclinical research. Administration of corticosterone via drinking water was chosen so that there is little to no stress associated with the actual process of administration of the glucocorticoid. Additionally, we wanted corticosterone levels to have a rhythmic nature rather than continuous stable administration, since this better recapitulates how endogenous corticosterone acts physiologically. This method of administration also provided us a simple opportunity to gather approximate corticosterone intake while monitoring daily water intake.

Aims of the study

Our research group has previously studied the effects of ketamine and N_2O in naïve rodents (Kohtala et al., 2019). mRNA sequencing data from our group's previous research showed that ketamine and N_2O treatment both upregulate several mRNAs implicated in MAPK signaling and synaptic plasticity (*Dusp1, Dusp5, Dusp6, Arc* and *Nr4a1*; Rozov et al., unpublished data). This project was designed to investigate this relationship further using an animal model of depression. The main goal of the current study was to continue the exploration of shared neurobiological mechanisms between rapid-acting antidepressants - in this case the widely accepted ketamine and the novel rapid-acting antidepressant N_2O. More specifically, we aimed to demonstrate behavioral and biochemical differences between naïve mice and a chronic corticosterone-induced animal model of depression, and to use this model to 1) investigate antidepressant-like effects of ketamine and N_2O and 2) elucidate the effects of ketamine and N_2O on five previously found shared gene markers.

Methods and materials

Animals

Male C57BL/6JHsd mice 7 weeks old at arrival (Envigo, Netherlands) were used. Mice were housed in sound-proof enclosures under controlled conditions (21°C, 12-h light-dark cycle, 6 AM lights on; Scantainers, Scanbur, Sweden) in the animal facility of the University of Helsinki, Finland, with free access to food and water unless mentioned otherwise. Mice were single-housed to ensure corticosterone drinking volumes stayed as consistent as possible between individuals. All treatments and behavioral tests excluding the saccharin preference test (SPT) were performed during the light phase (zeitgeber time, ZT 3-7). The experiments were carried out according to the guidelines of the Society for Neuroscience and were approved by the County Administrative Board of Southern Finland (License: ESAVI/5844/2019).

Pilot study

A pilot study was performed to estimate sample size and the dose of corticosterone (CORT) to be administered in the main study. Most previous studies have used doses of 25µg/ml or 100µg/ml. Both doses were tested, but only the higher dose of 100µg/ml had considerable effects on studied parameters (fig. 5). The pilot study was also used to observe the time frame of progression of the phenotype. The depressive phenotype was diminished to some extent after a week without CORT exposure (fig. 4). Hence, for this main study CORT was administered for 26 days and the decision made that administration will be continued during behavioral experiments.

Experimental plan

Mice were split into four groups: no corticosterone + vehicle (NO CORT VEH, *n*=15), corticosterone + vehicle (CORT VEH, *n*=13), corticosterone + ketamine (CORT KET, *n*=14) and corticosterone + nitrous oxide (CORT N2O, *n*=15). These groups were further split into two different cohorts: biochemical analysis (*n*=4-5 for each group) and behavioral analysis (*n*=9-11 for each group). See Appendix 1 for detailed *n* numbers. Since the weights of mice were spread evenly, all mice were randomly distributed to each group. A timeline of all procedures can be found in figure 2 and figure 3, and each procedure is outlined in further sections.

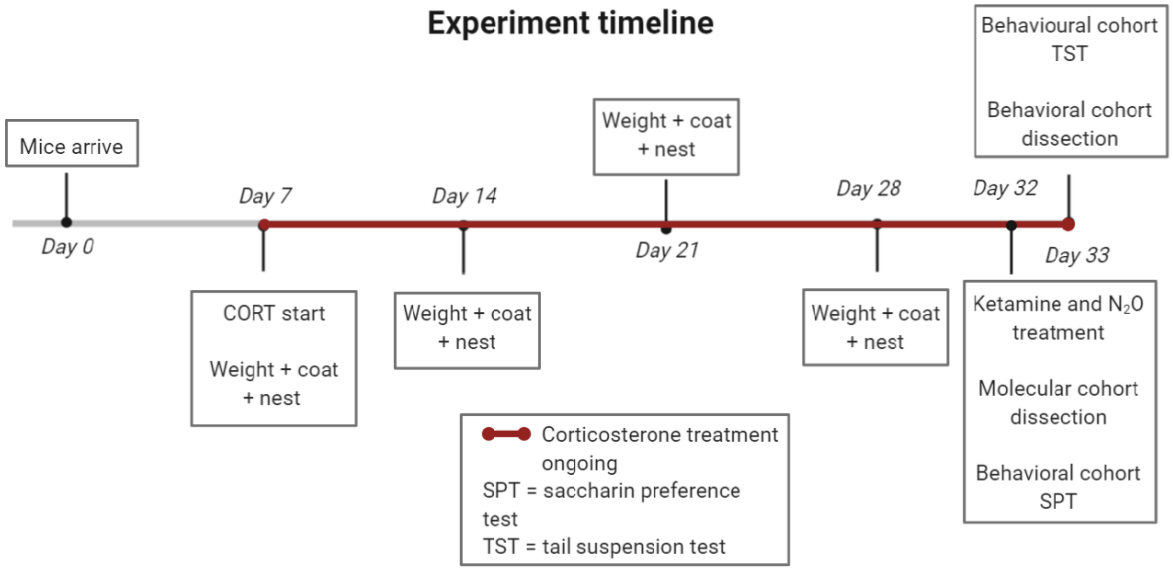

Figure 2. Schematic representation of the experiment timeline demonstrating the timing and duration of experimental procedures. After 7 days of habituation, CORT administration for a total of 26 days was started. Weights, coat state and nest quality were measured weekly. On the morning of day 32 mice were treated with vehicle (0.9% NaCl, i.p.), ketamine (10mg/kg, i.p.) or N_2O (50%, continuous administration for 1 h). Brain tissue was dissected from molecular cohort two hours after beginning of treatment. Saccharin preference test was performed for behavioral cohort in the beginning of the dark phase on the same day. On the morning of day 33 mice from behavioral cohort were subjected to tail suspension test after which brain tissue was dissected. CORT: corticosterone, N_2O: nitrous oxide.

Corticosterone treatment

Mice were habituated for a week before starting corticosterone treatment. Corticosterone (CORT; 46148-100MG, Sigma, USA) was first dissolved in 99,5% ethanol to ensure proper dissolution. This same method was used by Moda-Sava et al (2019), which was the main basis of this experiment plan. The mixture was then briefly sonicated and vortexed sufficiently to ensure corticosterone was properly dissolved. This CORT-ethanol solution was dissolved in animal facility provided tap water, so that the final concentration of ethanol was 1% and final concentration of CORT was 100µg/ml.

Mice were exposed to either CORT solution or 1% ethanol as control in place of drinking water for 26 days (fig. 2). Solutions were presented to mice in two 15 ml falcon tubes, with tips cut to a size which allowed drinking but prevented leakage. The weight of the drinking tubes was recorded daily at a similar time (ZT 5-7) in order to monitor daily drinking volumes of each mouse. To account for potential CORT degradation, all solutions were replaced with fresh ones every three days.

Drug treatments

Ketamine-HCl (Ketaminol®, Intervet International B.V., 511485) was diluted in physiological saline (0.9 % NaCl) solution and injected intraperitoneally (i.p.) 10mg/kg with an injection volume of about 10 ml/kg. This is a commonly used subanesthetic dose of ketamine associated with rapid antidepressant action in mice (Kohtala et al., 2019; Li et al., 2010; Moda-Sava et al., 2019) and the dose used in our previous mRNA sequencing study. Physiological saline was used for vehicle injections. Mice for biochemical analyses were dissected two hours after injection and those for behavioral analyses were dissected about 24 hours after injection (fig. 3).

Livopan®, a 50% nitrous oxide and 50% oxygen gas mixture (112115, AGA, Finland) was administered into airtight plastic chambers (width x length x height): 13x15x12 cm for 1 hour at a flow rate of 7 l/min. This concentration and duration of treatment was used in our previous mRNA sequencing study and the clinical study by Nagele et al., (2015). Physiological saline injections were also given intraperitoneally to the group treated with N_2O in order to control for effects of injection itself. Mice for biochemical analyses were dissected two hours after start of treatment and those for behavioral analyses were dissected about 24 hours after start of treatment (fig. 3).

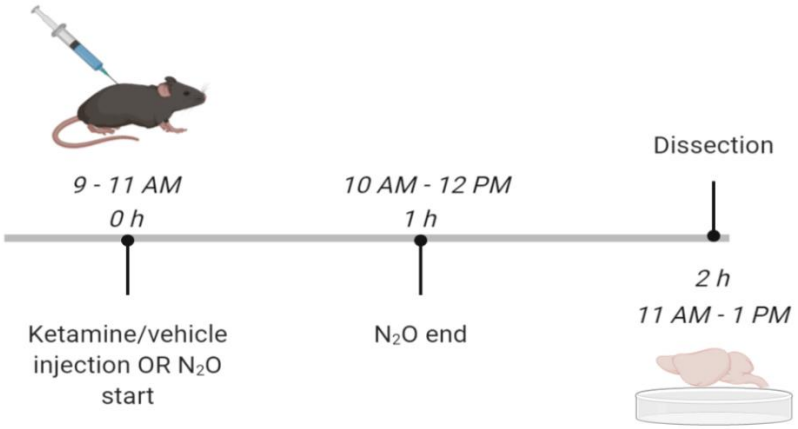

Figure 3. Schematic representation of the timeline for day 32 detailing vehicle, ketamine and N_2O treatment and molecular cohort dissection. Mice were treated with vehicle (0.9% NaCl, i.p.), ketamine (10mg/kg, i.p.) or N_2O (50%, continuous administration for 1 h) between 9 AM and 12 PM (ZT 3-6). Brain tissue was dissected from molecular cohort two hours after injection/beginning of treatment, between 11 AM and 1 PM (ZT 5-7).

Body weight and coat state assessment

Mice were weighed weekly at the same time of bedding change in order to avoid unnecessary stress and discomfort. This opportunity was also used to simultaneously assess their coat condition. The scoring of coat state was carried out by the assessment of eight different body

parts: head, neck, dorsal coat, ventral coat, tail, forepaws, hindpaws and genital region. A score of either 0 for a coat in a good state or a score of 1 for a dirty and unkempt coat were given for each of these areas. The total score for an individual mouse was obtained from the sum of the score of each of its body parts (Surget & Belzung, 2008). A mouse not showing a depression-like phenotype is assumed to exhibit normal grooming activity and thus a net coat condition with a low score, and vice versa.

Saccharin preference test

SPT examines the animal's motivation to experience hedonic pleasure evoked by sweet solutions (Klein, 1974). Since anhedonia is a hallmark symptom of MDD, drinking low volumes of saccharin compared to water indicates a depressive-like phenotype. Saccharin was chosen instead of sucrose due to saccharin having no caloric value, hence controlling for energy intake. Mice had been habituated to the presence of drinking tubes. On test day, drinking of the mice was restricted for eight hours (ZT 5:30-12:30) prior to testing. Saccharin solution was prepared immediately before testing, by dissolving saccharin sodium salt hydrate (S1002-500G, Sigma, USA) in standard animal facility drinking water to make 0.1% (w/v) solution. The mice were then given saccharin solution in one tube and water in the other tube for four hours (from ZT 12:30-16:30). At the end of this period, the tubes were taken away and experimental solutions were returned to mice. Water consumption and saccharin solution consumption were calculated by weighing the tubes before and after the four-hour testing period. Saccharin preference was determined by calculating the fraction (%) of saccharin water consumption divided by total water consumption.

Tail suspension test

Immobility in TST is a typical measure of behavioral despair modified by long-term administration of CORT and chronic stress (Moda-Sava et al., 2019). Latency to first bout of immobility in TST is sometimes used in addition to total immobility time as a co-indicator of depressive behavior. Its use is based on the same assumption of immobility being an expression of lack of escape related behavior. Mice with a depressive phenotype should "give up" quicker, hence have a shorter latency to their first immobility bout. Each mouse was brought into the test room for 30 minutes of acclimatization before the start of its test. Mice were recorded with Ethovision XT (Noldus, Netherlands) for 6 minutes while suspended from their tail 15 cm from the top of a compartment measuring 30 cm in height using lab tape. A small plastic tube was placed around the base of the tail to prevent tail climbing. The behavioral apparatus was first thoroughly cleaned with 70% ethanol and then water between

animals. Frame-wise changes in coordinates of a center of gravity were used as a proxy of subject's activity. To remove high frequency noise activity, data were off-line filtered by FIR low-pass filter (length: 5*sampling rate, cut-off: 0.5 Hz, transition: 0.5 Hz, attenuation: 60 dB) followed by normalization and assessment of total immobility time and latency by means of a custom-made Matlab routine (MathWorks, Nattick, USA).

Nest assessment

Impaired nest building is used as a measure of apathy, a deficit in goal-directed behavior, or disturbance in general activity in daily-living behavior (Planchez et al., 2019). A new nestlet was given to each mouse once a week along with bedding change. The quality of the nest built was assessed on a scale of 1-5 (1: untouched nestlet, 5: normal nest) weekly. A mouse not showing a depression-like phenotype is assumed to exhibit normal nest building activity.

Brain sample collection

Animals were euthanized at indicated times after treatment by rapid cervical dislocation followed by decapitation. No anesthesia was used due to its potential confounding effects on the analyses (Kohtala & Rantamäki, 2019). Bilateral medial prefrontal cortices and bilateral hippocampi were rapidly dissected on a cooled dish and stored at -80°C. The biochemical analyses in this project were conducted for the vmPFC, which was used for our previous mRNA sequencing study and is known to be an important region in the regulation of emotional behavior.

Quantitative RT-PCR

Total RNA of the samples was extracted using TRIzol reagent (15596018, Thermo Fischer Scientific, USA) according to manufacturer instructions with slight modifications. Frozen tissue was homogenized immediately after TRIzol addition and samples were kept at room temperature for 5 minutes to promote dissociation of nucleoprotein complexes. Chloroform (C2432, Sigma-Aldrich, USA) was added and the samples were vortexed for 15 seconds. Samples were again kept at room temperature for 5 minutes, after which they were centrifuged at 12,000 g for 15 minutes at 4°C. After centrifugation, all downstream preparation were performed on ice. The upper, aqueous phase containing the RNA was transferred to new tubes and isopropanol (AL03152500, Scharlau, Spain) was added for RNA precipitation. Samples were vortexed and incubated on ice for 10 minutes. After another 10-minute centrifugation at 12,000 g at 4°C, the supernatant was discarded carefully while leaving the RNA pellet at the bottom of the tube. The RNA pellet was washed twice with 1

ml of 75% ethanol and centrifuged at 12,000 g for 10 min at 4°C. Supernatant was once again carefully removed and the pellet left to air-dry for 5 minutes. The RNA pellet was dissolved in 30 µl of RNAse-free water, and carefully mixed by tapping the tube. The dissolved RNA was incubated in a heat block (Thermo Scientific, USA) set at 60°C for 10 minutes.

The concentration and purity of RNA was assessed with a Nanodrop 2000 Spectrophotometer. RNA samples with concentration > 100ng/µl, 260/280 ratio > 1.7 and 260/230 ratio > 1.8 were reverse-transcribed using the Maxima First Strand cDNA Synthesis Kit with dsDNase mix (K1672, Thermo Scientific, USA). Volume containing 1 µg of RNA was added to nuclease-free water so that the total volume was 12 µl for each sample. The same was done to an extra four random samples for future dilution series and control (no reverse transcriptase, NoRT) preparation. Master mixes were prepared for all samples containing reverse transcriptase (RT) and standards by combining the 5X Reaction Mix (contains reaction buffer, dNTPs, oligo(dT)18 and random hexamer primers), Maxima Enzyme Mix (contains the reverse transcriptase and RNase inhibitor) and nuclease-free water. Only 5X Reaction Mix and nuclease-free water was added to the NoRT sample. All samples were mixed and centrifuged briefly. RT Master Mix was then added to all RT samples, mixed and centrifuged. All samples were incubated for cDNA synthesis in a T100 Thermal Cycler (Bio-Rad, USA) according to the following: 10 minutes at 25°C (random hexamer primers annealing), 30 minutes at 55°C (oligo(dT)18 primers annealing and cDNA synthesis) and finally 5 minutes at 85°C (inactivation of reaction).

The cDNA was then prepared to be quantified using real-time PCR. Samples were diluted 1:10 and a dilution series was prepared from the extra three samples mentioned before. RT-PCR Master Mixes were prepared from the Maxima SYBR Green/ROX qPCR Master Mix (2X) (K0221, Thermo Scientific, USA) by combining 2X Maxima SYBR Green qPCR Master Mix, 10 µM Forward primer, 10 µM Reverse primer and nuclease-free water so that the final volume was 6 µl for each well used. The primers used to amplify specific cDNA regions of the transcripts are shown in Table 1. Primers for *Dusp1*, *Dusp5*, *Dusp6* and *Nr4a1* were designed *de novo* and characterized for selectivity and specificity. DNA amplification reactions were run in triplicates. After pipetting the RT-PCR Master Mix into a 384-well plate, 2 µl of diluted cDNA templates were added into each well. 2 µl of nuclease-free water was added instead of cDNA templates in the well for a second control (no-template control). A clear Microseal Film was applied to the plate and it briefly centrifuged. RT-PCR was performed for 45 cycles on the Lightcycler® 480 System (Roche, Germany). Relative

quantification of template was performed by calculating C_q values using the second derivative and dilution series method, with cDNA data being normalized to the *Gapdh* and *ß-actin* levels.

Table 1. Primers used for quantitative RT-PCR.

Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)	References
Gapdh	GGTGAAGGTCGGTGTGAACGG	CATGTAGTTGAGGTCAATGAAGGG	(Karpova et al., 2010)
β-actin	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT	Primer bank ID 6671509a1
Arc	AAGTGCCGAGCTGAGATGC	CGACCTGTGCAACCCTTTC	Primer bank ID 9055166a1
Dusp1	GGGAGAGTGTTTGTTCATTGCC	TCTGCTTCACAAACTCAAAGGC	Rozov et al., unpublished
Dusp5	CAACTTTGGCTTCATGGGACAG	AGGGCTCAGTGTCTGTAAATGG	Rozov et al., unpublished
Dusp6	ACAAAACTGGGCACCTTCATTC	TAGGGAAAGCGACACAGAAGTC	Rozov et al., unpublished
Nr4a1	GGGAGTGTGCTAGAAGGACTG	AGCTTGAATACAGGGCATCTCC	Rozov et al., unpublished

Statistical Analyses

Pilot study

Pilot study data are presented as either median ± SD or box plots with median, interquartile range, and minimum and maximum values. All statistics were calculated using R software (version 3.5.2). Data were examined for outliers either with Grubb's test or Dixon's test depending on whether they fit assumptions of normality or not. Due to the data violating assumptions for parametric analyses, differences in coat state scores and saccharin preference were determined by Kruskal–Wallis test followed by Dunn's Multiple Comparison *post-hoc* test with Bonferroni correction if significant. Immobility in TST results were analyzed by univariate one-way ANOVA. P values <0.05 were considered to be statistically significant.

Main study

Main study data are presented as box plots with median, interquartile range, and minimum and maximum values. All statistics were calculated using R software (version 3.5.2). Data were examined for outliers either with Grubb's test or Dixon's test depending on whether they fit assumptions of normality or not. Only one value in SPT main study results was deemed an outlier, but its removal did not change significance of results, hence it was included in the analysis. Differences among experimental groups in qPCR analysis were determined by one-way analysis of variance (ANOVA), followed by Tukey *post-hoc* test with Bonferroni correction if ANOVA was significant. Differences among experimental

groups in behavioral tests were determined by either Kruskal–Wallis test or one-way multivariate ANOVA (MANOVA). Due to the data violating assumptions for parametric analyses, differences in coat and nest assessment were determined by Kruskal–Wallis test followed by Dunn's Multiple Comparison *post-hoc* test with Bonferroni correction if significant. A one-way MANOVA was conducted for the continuous numerical data to test the hypothesis that there would be one or more mean differences between experimental groups (independent variables, IVs) and different behavioral test results (dependent variables, DVs). This was followed by univariate one-way ANOVAs in order to determine how each dependent variable differs for the independent variable. Finally, if the effect was found to be significant, this was followed by Tukey *post-hoc* test with Bonferroni correction. P values <0.05 were considered to be statistically significant.

Results

Pilot study

A pilot study was performed to observe whether chronic oral corticosterone administration produces depressive behavior. A mouse not showing a depressive-like phenotype is assumed to exhibit normal grooming activity and thus a net coat condition with a low score. The increase in coat score after 25 days of higher dose CORT administration [$H(3) = 10.51$, $p = 0.0006$; NO CORT vs. CORT HIGH $p = 0.0036$, fig. 5a] therefore indicates an unkempt coat and possibly one manifestation of depressive behavior. Coat state worsened gradually as CORT administration progressed, with CORT HIGH group scores increasing to a larger extent compared to NO CORT or CORT LOW group (fig. 4a). Coat state started progressively but slowly reverting to baseline when CORT administration was stopped after 26 days. A similar phenomenon occurred with saccharin preference, wherein one week of CORT administration was not enough to produce observable differences but after that saccharin preference was progressively reduced in the CORT HIGH group (fig. 4b). After 24 days of CORT administration, saccharin preference was significantly reduced in the CORT HIGH group [$H(3) = 7.768$, $p = 0.0117$; NO CORT vs. CORT HIGH $p = 0.0172$, fig. 5b]. This indication of anhedonia was also reverted after CORT administration ended (fig. 4b). TST was performed after 26 days of CORT administration. Immobility duration seemed to be increased after the higher dose of CORT, but possibly due to high variability in results of both control and CORT LOW groups, this increase was not significant [$F(2, 12) = 2.160$, $p = 0.1580$, fig. 5c]. TST was not repeated after the week of CORT withdrawal due to possible habituation affecting the reliability of results.

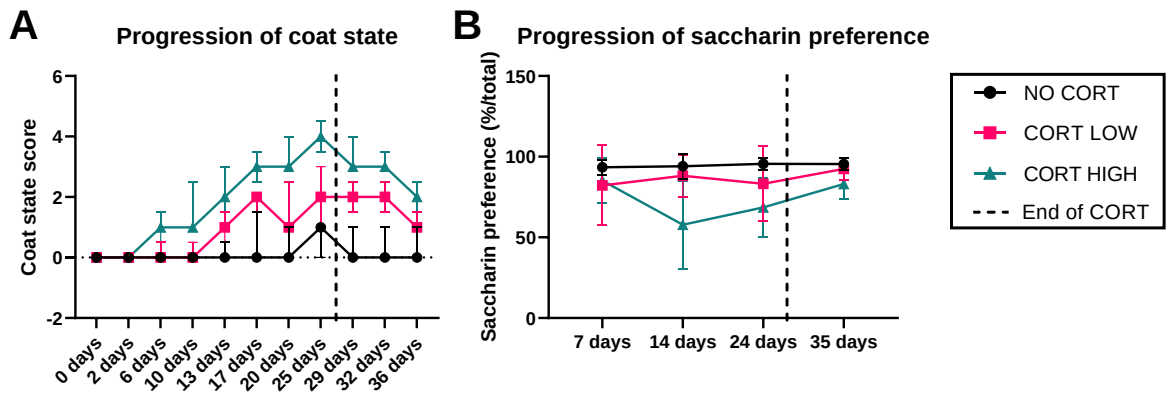

Figure 4. Chronic administration of a higher dose (100µg/ml) of CORT caused a progressive deterioration of coat state and reduction in saccharin preference during the pilot study. (A) Progression of coat state score during and after CORT administration phase. (B) Progression of saccharin preference at the beginning of the dark phase during and after CORT administration phase. Data depicted as median ± SD. NO CORT: control group, CORT LOW: corticosterone 25µg/ml, CORT HIGH: corticosterone 100µg/ml.

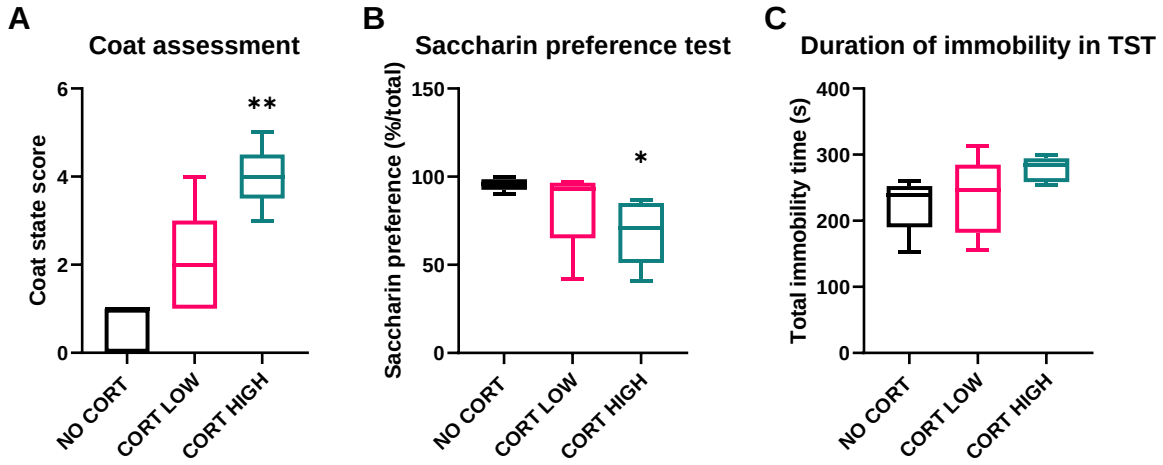

Figure 5. Chronic administration of a higher dose (100µg/ml) of CORT led to significant worsening of coat state and decreased saccharin preference, but not to changes in immobility during the pilot study. (A) Coat state score after 25 days of CORT administration. (B) Saccharin preference after 24 days of CORT administration. (C) Total immobility time in TST at the end of CORT administration phase (after 26 days of CORT). Boxplots depict the median, interquartile range, and minimum and maximum. Compared to control group (NO CORT) = *<0.05, **<0.01 (for detailed statistical analyses and *n* numbers see Appendix 1). NO CORT: control group, CORT LOW: corticosterone 25µg/ml, CORT HIGH: corticosterone 100µg/ml, TST: tail suspension test.

Main study

In order to test the hypothesis that there would be one or more mean differences between different behavioral test parameters and experimental groups, a MANOVA analysis was conducted on those main study results which fit assumptions of parametric testing. According to the analysis, CORT administration and/or treatments lead to a change in the combined

behavioral phenotype consisting of saccharin preference, duration of immobility in TST and latency to immobility in TST [Pillai's Trace = 0.65, F (3,35) = 3.23, p = 0.0017]. Therefore, univariate one-way ANOVAs were performed in order to determine which experimental variables caused changes in which behavioral parameters.

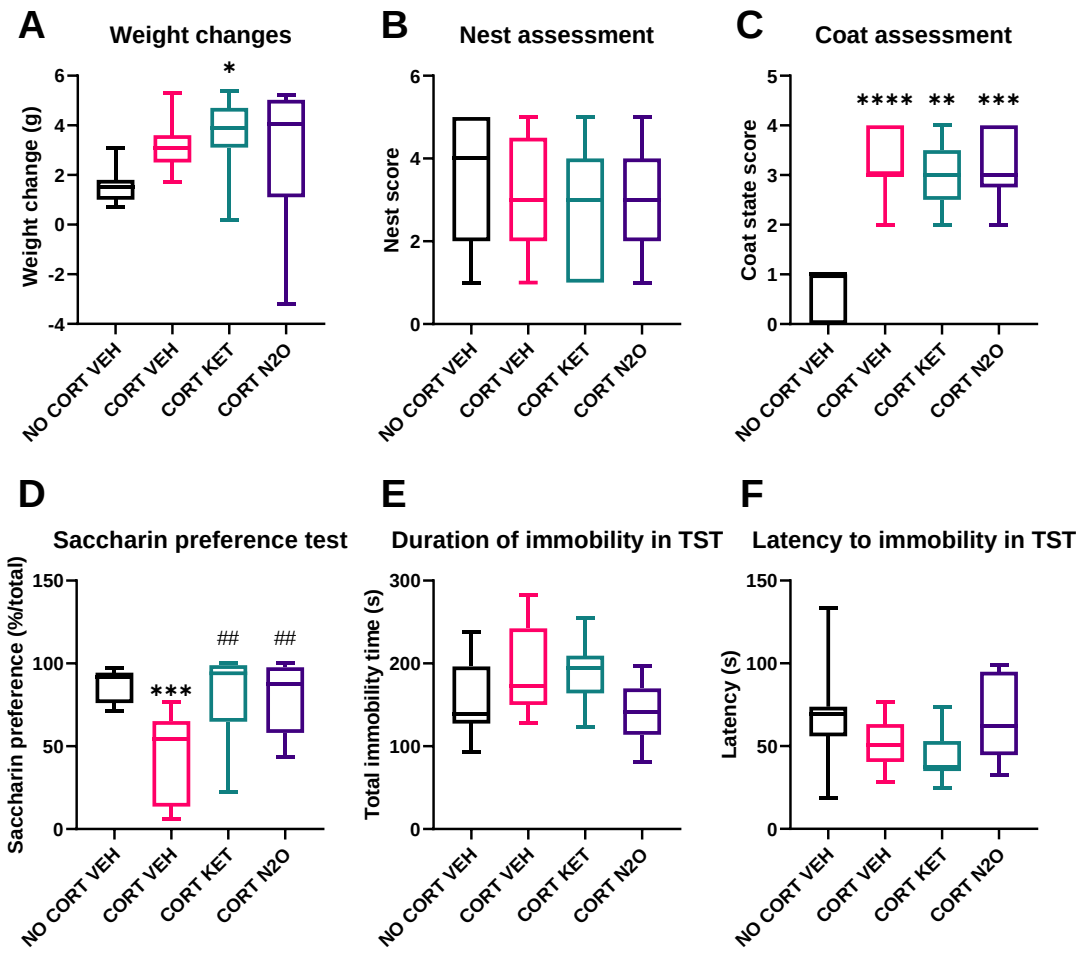

Figure 6. The effects of chronic CORT administration and ketamine or N_2O treatment on the behavioral phenotype of C57BL/6JHsd mice in the main study. (A) Total body weight change of mice from the beginning of the experiment to right before treatment. (B) Nest assessment before treatment. (C) Coat assessment 24 h after treatment. (D) Saccharin preference during the start of the dark phase on the same day as treatment. (E) Total immobility time in TST 24 h after treatment. (F) Latency to the first bout of immobility in TST 24 h after treatment. Treatments consisted of vehicle (0.9% NaCl, i.p.) or ketamine (10mg/kg, i.p.) injection or continuous administration of N_2O (50%) for 1 h. Boxplots depict the median, interquartile range, and minimum and maximum. Compared to control group (NO CORT VEH) = *<0.05, **<0.01, ***<0.005, ****<0.001. Compared to CORT VEH = ##<0.01 (for detailed statistical analyses and *n* numbers see Appendix 1). N_2O: nitrous oxide, TST: tail suspension test.

No clear impact of chronic exogenous corticosterone administration on body weight

A change in body weight is considered one possible symptom of depression. No clear relationship between body weight and depression exists however, as exemplified by the

inconsistent results of previous clinical and preclinical research (American Psychiatric Association, 2013; Planchez et al., 2019). In this study, body weight was not measured after ketamine or N_2O treatments; hence this comparison of body weight changes shows only the effect of chronic CORT administration on body weight. Total body weight mostly increased in all experimental groups during the study (fig. 6a). Kruskal-Wallis analysis implicated that CORT caused a significant increase in body weight [$H(4) = 11.494$, $p = 0.0093$]. However, Dunn's *post hoc* analysis with Bonferroni correction showed a significant increase in body weight only between the control group and CORT KET group ($p = 0.0108$). No clear effect of chronic CORT on body weight could therefore be deduced.

Chronic corticosterone produces some behaviors of a depressive-like phenotype

In order to test whether chronic oral administration of CORT produces depressive-like behavior, we implemented tests measuring behavioral symptoms of depression such as lack of self-care and anhedonia. Nest assessment showed no significant differences between any groups [$H(4) = 2.61$, $p = 0.4551$, fig. 6b], suggesting that chronic CORT does not affect nest building activity. Conversely, coat state was clearly worsened indicated by increased scores in CORT administered groups [$H(4) = 25.24$, $p < 0.0001$, fig. 6c]. Furthermore, Dunn's *post hoc* analysis with Bonferroni correction revealed significant increases in coat state scores between the control group and all three CORT treated groups (CORT VEH vs. control $p = < 0.0001$, CORT KET vs. control $p = 0.0017$, CORT N_2O vs. control $p = 0.0005$). Hence, chronic CORT worsened coat state indicating lack of grooming and consequently a manifestation of a depressive-like phenotype. This was not, however, reversed by neither ketamine nor N_2O treatment.

Reduced saccharin preference depicts another form of depressive-like behavior. ANOVA analysis of saccharin preference results depicted significant changes caused by CORT administration and/or treatments [$F(3, 35) = 8.001$, $p = 0.0003$]. Tukey *post hoc* analyses with Bonferroni correction showed a significant decrease in saccharin preference between the control group and CORT VEH group ($p = 0.0004$, fig. 6d). This decrease was significantly reverted by both ketamine and N_2O treatments (CORT KET vs. CORT VEH $p = 0.0039$, CORT N_2O vs. CORT VEH $p = 0.0046$), suggesting that both treatments produced antidepressant-like effects.

Neither chronic corticosterone, ketamine nor N_2O produced significant changes in lack of escape-related behavior in the tail suspension test

Duration of immobility in TST is expected to be increased and latency to immobility decreased in a depressive-like phenotype. In this study, ANOVA analysis of total immobility time in TST indicated a significant difference between groups [F (3, 35) = 3.119, p = 0.0383]. However, Tukey *post hoc* analyses after Bonferroni correction did not show any significant differences between groups (fig. 6e). Similarly, latency to immobility did not show any significant differences between groups, as tested by ANOVA analysis [F (3, 35) = 2.719, p = 0.0593, fig. 6f]. Hence this form of depressive behavior was not clearly produced by chronic CORT, and no conclusions regarding rescue of this behavior could be made.

N_2O treatment increased both *Dusp1* and *Dusp6*, but not *Dusp5* mRNA expressions

ANOVA analysis of both *Dusp1* and *Dusp6* implicated a significant change in mRNA expression in the vmPFC between experimental groups [F (3, 14) = 4.260, p = 0.0247 and F (3, 14) = 4.277, p = 0.0244 respectively]. Tukey *post hoc* analyses with Bonferroni correction showed that N_2O significantly increased both *Dusp1* (CORT VEH vs. CORT N_2O p = 0.0323, fig. 7a) and *Dusp6* expression (NO CORT VEH vs. CORT N_2O p = 0.0290, fig. 7c). No other comparisons, notably not even between CORT VEH and control, showed significant differences (fig. 7). No significant changes in *Dusp5* expression were observed (fig. 7b).

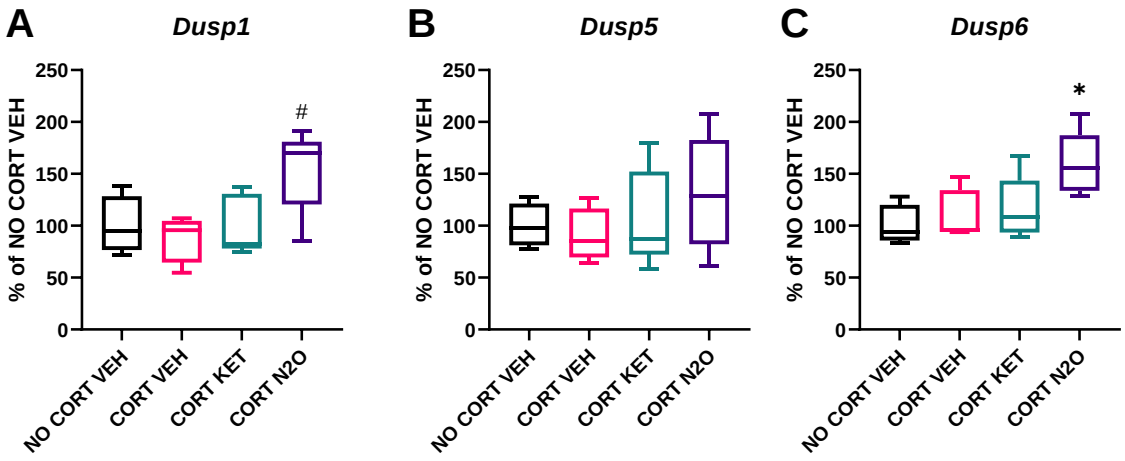

Figure 7. Levels of *Dusp1* (A), *Dusp5* (B) and *Dusp6* (C) mRNA in the vmPFC 2 h after vehicle (0.9% NaCl) or ketamine (10mg/kg, i.p.) injection, or start of N_2O treatment (50%, continuous administration for 1 h) relative to control group. Boxplots depict the median, interquartile range, and minimum and maximum. Compared to control group (NO CORT VEH) = *<0.05. Compared to CORT VEH = #<0.05 (for detailed statistical analyses and *n* numbers see Appendix 1). vmPFC: ventromedial prefrontal cortex, N_2O: nitrous oxide.

Arc and *Nr4a1* mRNA expressions were increased by N_2O treatment

ANOVA analyses of *Arc* and *Nr4a1* suggested a significant change between experimental groups [F (3, 14) = 4.105, p = 0.0277 and F (3, 14) = 4.904, p = 0.0156 respectively]. Tukey *post hoc* analyses with Bonferroni correction showed a significant increase of both targets after N_2O treatment (NO CORT VEH vs. CORT N_2O: *Arc* p = 0.0321 and *Nr4a1* p = 0.0182), but no other significant differences were observed (fig. 8). The increases produced by N_2O treatment were very large with some samples, even threefold or fourfold, but the significance was diminished by variability in the group. As with *Dusp*s (fig. 7), chronic CORT administration did not seem to produce any changes in amount of *Arc* and *Nr4a1* mRNA (fig. 8).

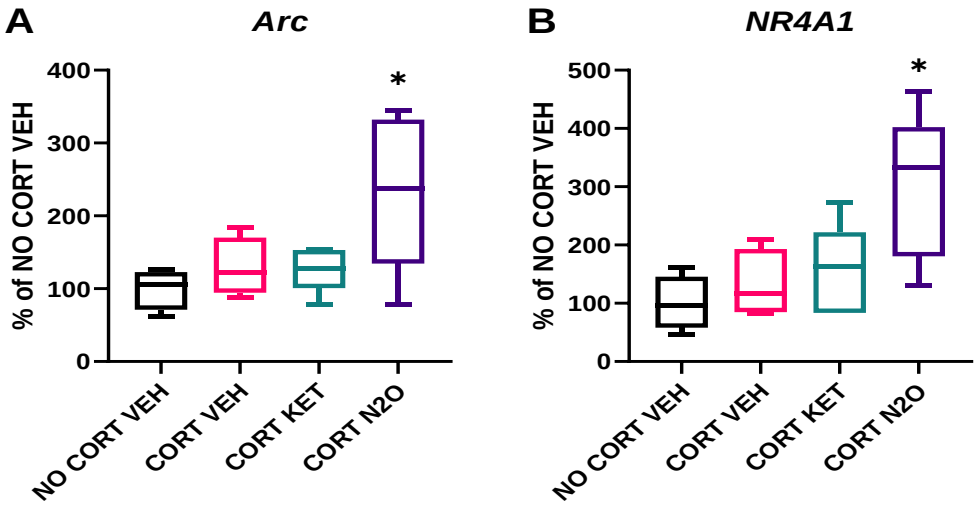

Figure 8. Levels of *Arc* (A) and *Nr4a1* (B) mRNA in the vmPFC 2 h after vehicle (0.9% NaCl, i.p.) or ketamine (10mg/kg, i.p.) injection, or start of N_2O treatment (50%, continuous administration for 1 h) relative to control group. Boxplots depict the median, interquartile range, and minimum and maximum. Compared to control group (NO CORT VEH) = *<0.05 (for detailed statistical analyses and *n* numbers see Appendix 1). vmPFC: ventromedial prefrontal cortex, N_2O: nitrous oxide.

Discussion

One goal of this study was to explore the use of an animal model of depression induced by chronic oral administration of corticosterone. The results show a clear distinction between the depressive and control phenotype groups in both SPT and coat assessment. The decrease in saccharin preference exhibited by the CORT VEH group can be considered an indication of anhedonia, which is regarded as a key symptom of depressive behavior, in both animal models and humans. An unkempt coat condition is used as a hallmark of depressive behavior in animal models (Surget & Belzung, 2008). This also makes sense, because it has exceptional translational value – i.e. the same phenomenon, lack of self-care, can be seen in

many patients with MDD. The progressive worsening of coat state observed in the pilot study (fig. 4) exemplifies the time-dependent nature of the phenotype. Even though coat state is a widely used indicator of depression-like behavior, this study shows it is not necessarily the best parameter for studying efficacy of rapid-acting antidepressants. CORT treatment worsened coat condition, but this was not rescued by either treatment. A possible explanation why these treatments did not reverse the state of coat lies in the fact that coat state is a physical parameter that worsens over time. Consequently, it might not be possible for coat state to return to baseline in such a time frame even if grooming behavior is normalized. In experiments conducted with traditional antidepressants, coat state changes on average only after two weeks of treatment. A glyoxylase 1 inhibitor, which regulates levels of cytotoxic glycolysis byproducts and has been proposed to act as a rapid-acting antidepressant, reversed deterioration of coat state in 5 days (McMurray et al., 2018). Whether this is due to the nature of these antidepressants or the fact that it is simply not possible to rescue coat state in a shorter time period is uncertain. The results of our pilot study also demonstrated this. After the termination of CORT administration, coat state started improving but this change was slow, and even after 10 days none had reverted to baseline condition. A solution to this could be the use of splash test in addition to basic coat assessment. In a splash test 10 % sucrose solution is squirted on the dorsal coat of mice and because of the solution's viscosity, it dirties the mouse fur and provokes grooming behavior (Surget & Belzung, 2008). The latency to initiate grooming behavior as well as the frequency and the duration of grooming in five minutes is measured. This presents direct information about the actual act of grooming and not just the result of it.

Another goal of this study was to investigate the extent of antidepressant-like effects of ketamine and N_2O. Both N_2O and ketamine's ability to reverse anhedonia observed in SPT performed less than 12 hours after treatment can be considered a rapid antidepressant effect. There were no significant differences in immobility nor latency to immobility in TST between groups after *post hoc* comparisons. One would expect duration of immobility to increase and latency to immobility decrease after chronic CORT administration, and for these changes to be brought back to baseline after ketamine or N_2O treatment. TST is often used to screen for antidepressant drugs, which is why it is quite unexpected that no significant differences were observed between any experimental groups. Unfortunately, the variance in both immobility and latency to immobility results was rather large, which could partly

explain why no significant results were reached. Nonetheless, this means no confident conclusions can be made concerning this aspect of depressive-like behavior.

Most interestingly, ketamine clearly produced a significant antidepressant effect in SPT, but its effect in TST was even less clear than that of N_2O. Previous studies have shown that ketamine has lasting antidepressant effects measured by TST at 24 hours after treatment (Moda-Sava et al., 2019), which contradicts our results. The results of TST generally were a lot more varying compared to SPT, which could partially explain this discrepancy. The method might be more vulnerable to small fluctuations such as physiological stress reactions to injections between individual mice. The way TST raw data was processed may also have affected the results. In our pilot study, chronic administration of the same dose of CORT (100µg/ml) produced a clearer increase in immobility time, although this also was not significant (fig. 5c). In the pilot study, we used Ethovision-processed data for statistical analysis, whereas here we processed the raw data ourselves (see Methods) due to the presence of high frequency noise in the data provided by Ethovision. Even so, we did choose five mice at random to score manually and compared those observations to the results calculated from our processed data. Immobility times and latencies were higher than when observed manually, but the differences stayed consistent between mice. More importantly, the reliability of TST results in this main study is undermined by the fact that no general locomotor activity was recorded. Baseline locomotor activity can greatly influence immobility in TST, which can lead to falsely observed or false lack of differences between groups. Finally, one notable factor that could contribute to variation in behavioral results is the time between rapid-acting antidepressant treatment and behavioral testing, and possibly even time of treatment administration itself. To examine this, the results of SPT and TST were plotted against these factors (see Appendix 2). A negative correlation with SPT results and the time between treatment and testing can be seen to some extent, but a clear trend cannot be deduced from TST. Hence, this does not provide an explanation for the large variation of TST results.

The final goal of this study was to further explore the effects of ketamine and N_2O on previously observed shared molecular markers, but in an animal model of depression. This study demonstrates that N_2O significantly upregulates *Dusp1, Dusp6, Arc* and *Nr4a1* mRNA expression in the vmPFC after a 1-hour treatment and following 1-hour recovery period. *Dusp5* levels were not significantly changed. Ketamine did not produce similar results in any of the targets. The variation in qPCR results is quite high, which could be due to purity of

mRNA varying from sample to sample, and the sample size being small (n = 4-5). From this study by itself, it is not possible to say whether the lack of antidepressant effects from ketamine is due to this specific dose not producing such robust antidepressant effects as N_2O, and thus we see no changes in mRNA markers, or if ketamine just does not affect these markers in question. This dose of ketamine was chosen because it has often been shown to produce rapid antidepressant effects (Beurel et al., 2011; Li et al., 2010; Maeng et al., 2008), which contradicts the former. Additionally, ketamine did significantly increase saccharin preference in this study. However, the latter option also conflicts with not only our previous mRNA sequencing results (see Introduction; Rozov et al., unpublished data) but with other studies as well. A single dose of ketamine has been shown to transiently upregulate *Arc* in the PFC 1 hour after administration (Kohtala et al., 2019), with levels returning to baseline after 6 hours (Li et al., 2010). Ketamine also normalizes the upregulated protein levels of DUSP1 at 30 minutes and 72 hours after injection, and the downregulated phosphorylation of MAPK3 30 minutes after injection in the anterior cingulate cortex of a chronic pain induced model of depression (Humo et al., 2020). Thus, the results from this study differ from the results of most previous studies in two ways: 1) ketamine did not produce any molecular changes in these markers, and 2) *Dusp* levels are increased by N_2O, not brought back to control levels as has been shown with both ketamine and traditional antidepressants. Even still, N_2O treatment did partly produce similar effects to ones previously reported with rapid-acting antidepressants. Both *Arc* and *Nr4a1* expression was increased (Choi et al., 2015; Kohtala et al., 2019; Li et al., 2010), although upregulated *Nr4a1* has only been demonstrated *in vitro* (Choi et al., 2015). Additionally, our previous mRNA sequencing study showed upregulation of these target markers by N_2O (Rozov et al., unpublished data), just as in this study.

Interestingly the molecular markers showed no significant changes between control and CORT VEH groups. Previous studies have shown that *Dusp*s and *Nr4a1* are upregulated in animal models of depression, although the results vary depending on brain region and method used to create the model (Duric et al., 2010; Gourley, Wu, & Taylor, 2008; Jeanneteau et al., 2018). *Arc* has been found to be downregulated in the frontal cortex and CA1 area of the hippocampus after chronic mild stress and in a genetic animal model of depression (Elizalde et al., 2010). MAPK pathway inhibitors cause depressive-like behavior (Duman et al., 2007; Duric et al., 2010), the phosphorylation of MAPKs is reduced in depressed patients and animal models of depression (Dwivedi et al., 2001; Meller et al., 2003) and MAPKs and

DUSPs are known to function in a negative feedback loop. Based on this it would be expected that if MAPK activity has been decreased for a longer time, then DUSPs are not upregulated either, as is the case in this study. Nonetheless, MAPK levels were not measured here, so no conclusions regarding this can be made. Furthermore, inhibited MAPK activity is a common finding among depressed patients and animal models, where it has arguably been this way for a longer time. Even still, *Dusp* expression is upregulated in these cases. This would suggest that *Dusps* are sustained by not only MAPKs, but other mechanisms as well, which keeps MAPK phosphorylation levels low. Indeed, the relationship between DUSPs and MAPKs can be regulated differently depending on the condition of specific cells and networks. MAPK3 can trigger DUSP1 proteolysis via the ubiquitin-proteasome pathway, hence achieving a positive feedback loop by decreasing its phosphatase activity (Lin et al., 2003). Therefore, depending on the situation, DUSP activity can be modulated in either direction by the MAPK pathway. Another level of complexity is created by the fact that DUSPs are not only regulated on an mRNA level (Pérez-Sen et al., 2019). The results of this study could simply indicate that chronic CORT administration does not lead to these markers being regulated on a transcriptional level, but rather on a post-translational or epigenetic level.

It is still common to think of antidepressants as reversing a molecular problem or bringing specific protein levels back to normal, which then leads to rescue of depressive behavior. However, as the results of this study show, their actions are far more complex. N_2O led to antidepressant-like behavioral effects and caused significant upregulation in selected mRNAs that modulate MAPKs, but it did not reverse expression of any of the targets tested, since the CORT VEH group did not differ from the control group. The observed difference of the results from this thesis to previous studies could stem from timing. It has been shown that rapid-acting antidepressants, specifically ketamine, cause cortical excitation (Kohtala et al., 2019). Here it is shown in the form of *Arc* upregulation from N_2O treatment. MAPK1/3 phosphorylation is another sign of excitation, and it has been shown to be upregulated after treatment with both ketamine and N_2O (Kohtala et al., 2019; Li et al., 2010). However, this upregulation is transient, and soon after phases into downregulation. Excitation of the cortex has also been shown to lead to endogenous BDNF release, and BDNF is necessary for *Dusp1* mRNA induction (Jeanneteau et al., 2010), further suggesting a possible need for neuronal excitation for the activation of these pathways. If N_2O causes cortical excitation and thus MAPK pathway activation, this might cause a homeostatic need for *Dusp* levels to increase and lessen that activation, which ultimately leads to MAPK downregulation. Studies that

have shown downregulation of *Dusp*s after rapid-acting antidepressant treatment have had brain tissue dissected for biochemical analyses either 7 hours after ketamine injection or after traditional antidepressant treatment – i.e. a longer period of time (Duric et al., 2010; Orozco-Solis et al., 2017). In this study we dissected tissue for analysis 2 hours after treatment, so it could be that dissection was performed during the homeostatic rise of *Dusp*s and that their expression may have been downregulated after. *Arc* is not only a marker of neuronal excitability, but also an important player in synaptic plasticity, because it regulates AMPAR endocytosis. Upregulation of *Arc* as seen in this study with N_2O treatment may reflect the importance of homeostatic action as well. As mentioned previously, BDNF elevates *Arc* mRNA levels and this elevation is blocked by MAPK pathway inhibitors (Rao et al., 2006). This leads to an interesting contradiction, in that N_2O treatment upregulates *Arc* in our study, where we assume MAPK activation to be lowered due to inhibition from upregulated *Dusp* expression.

As discussed previously, *Dusp*s and *Nr4a1* are often upregulated in depressed patients. Additionally, acute stress causes transient MAPK pathway activation and *Arc* upregulation similarly to rapid-acting antidepressants. How do the phenomena demonstrated in this study then differ from the observations of depressed patients and acute stress? At first glance, the molecular response produced by N_2O seems identical to what has been exhibited in depressed patients and animal models of depression. This indicates that the existing difference between these two states, rapid-acting antidepressant and depressed behavior, is more physiologically complex than just mRNA and protein levels. We hypothesize that the answer lies in the timing and homeostatic aspect of the brain's responses. N_2O and other rapid-acting antidepressants present a challenge for the brain, which then demands a homeostatic response. This response leads to synaptic changes, which have consistently been shown to be integral for sustained antidepressant effects. In support of this, many of the pathways and markers discussed in this thesis have straightforward connections to synaptic plasticity and microtubule organization. More importantly, this study is not the only one presenting contradicting results to previous research. Kodama et al. (2005) showed that *Dusp1* and *Dusp6* mRNA was increased in the frontal cortex of rats 1 hour after an electroconvulsive shock. Agreeing with the hypothesis presented in this thesis, no upregulation in either marker was observed at a later 6-hour time point.

An important question to be answered in future research is what systemic neurobiological level changes does rapid-acting antidepressant treatment cause that lead to amelioration of

depressive symptoms even though the expression of the majority of these gene markers, at least 2 hours after treatment, are comparable to those seen in depressed patients and animal models of depression. Analysis of different levels of regulation of *Dusp*s, such as protein and phosphorylation levels with western blotting should be incorporated into future studies. This thesis brought up the possible importance of timing when it comes to analysis of biochemical effects of rapid-acting antidepressants, hence the progression of MAPK pathway activation and simultaneously *Dusp* expression should be focused on. There exists little research particularly on *Dusp* activation kinetics in relation to rapid-acting antidepressants. N_2O exhibits fast pharmacokinetics, which makes it a valuable tool for studying the time-dependency of these phenomena. One approach to study this would be to regulate the MAPK pathway pharmacologically using compounds known to inhibit its function, such as PD184161, at different timepoints relative to N_2O treatment. The most critical timepoints to compare would be injection of inhibitor immediately before and after treatment. Dissection times should also be varied, in order to confirm the speculation that the observed differences in *Dusp* regulation of this study to previous ones is due to different times between treatment and dissection. In order to further elucidate the relationship between cortical excitation and the behavioral and biochemical effects of rapid-acting antidepressants, electroencephalography (EEG) should be used for proper verification of excitation and observation of how the brain responds. This would allow analysis of the phenomenon on a more integrated level, especially when EEG events are compared with biochemical analyses from different timepoints.

In future studies corticosterone levels circulating in the blood should be measured. Though measurement of drinking volumes can give some reference on the corticosterone amount each mouse has ingested, there are still factors that can affect how much makes it into the circulation. Measuring corticosterone directly combats this and would allow for better analysis of the correlational relationship between depressive behavior and amount of corticosterone the mouse was exposed to. One limitation of this study is the fact that corticosterone was dissolved in ethanol. The final concentration of ethanol was only 1% and it was controlled for by having the control group drink tap water with the same concentration of ethanol. Nonetheless, this results in nondescript unknowns possibly affecting the biochemistry of the brain and behavior. Corticosterone hemisuccinate could be used instead to avoid this, since it is less hydrophobic and can be dissolved in plain tap water. However, this does not necessarily erase the problem of introducing another foreign chemical into the

experimental design. This might still be a better option, since it is well-known that ethanol greatly affects the brain. Another significant limitation of this study is the fact that there was no proper control group for the effects of N_2O. N_2O was administered in gas form and the mice were placed in a novel box for the duration of treatment. The novelty of both incoming air pressure and a new environment can greatly affect the physiology and subsequently the behavior of these mice. Hence, this form of administration should be controlled for by having a separate cohort of animals in the same environment being administered a control gas such as ambient air. Previous studies by our research group have shown that this does not significantly impact the biochemistry or behavior of mice (Kohtala et al., 2019; Rozov et al., unpublished data). Still, it does not change the fact that the lack of this control group in the present study makes it difficult to analyze the results confidently. Thankfully, this is a problem that can and should be fixed in future studies.

This thesis aimed to explore the shared behavioral and biochemical effects of rapid-acting antidepressants ketamine and N_2O in a chronic CORT-induced animal model of depression. This study has high novelty value, due to the study of N_2O in a potential mouse model of depressive behavior. These preliminary results suggest that N_2O could be a suitable candidate for rapid treatment of depression, since it reversed anhedonia observed in SPT. The antidepressant-like effects of N_2O were accompanied by significant upregulation of *Arc, Dusp1, Dusp6* and *Nr4a1*. No notable effects in target mRNAs were observed by sole chronic CORT administration or by ketamine treatment. This study produced both similarities and differences in results to previous research. Differences in time between treatment and tissue dissection is presented here as the possible cause of conflicting molecular results, particularly the ones observed in *Dusp* expression. We suggest that the action of rapid-acting antidepressants, in this study mostly N_2O, is based on a homeostatic response of the brain to a presented challenge. Here this challenge would be cortical excitation previously been shown to be caused by N_2O, which leads to activation of pathways such as MAPK and subsequent *Arc, Dusp* and *Nr4a1* signaling. The level of expression of these molecular markers would then depend on which phase this response is in. While the chronic CORT-induced animal model demonstrated some behaviors typical of a depressive-like phenotype, it did not lead to any changes in the measured mRNA targets. This should be studied in more detail, since it contradicts data gathered from other animal models of depression and depressed patients. Furthermore, future studies would benefit from detailed validation of this model due to its potential in controlling for behavioral variability and thus reducing the number of animals

needed for preclinical research. Overall, if the findings of this thesis are replicated, it could be one of the first steps in the search for the mechanisms underlying the potential antidepressant effect of N_2O, how these molecular markers are related to its action and how the actions of ketamine differ from these.

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APPENDICES

Appendix 1: Details of all statistical analyses

Figure 5			n	Statistical test		Significance	
A	Coat assessment		n = 5, 5, 5, 5	Kruskal-Wallis one-way ANOVA		H(3) = 10.51	p = 0.0006
		NO CORT vs. CORT LOW	n = 5, 5	Dunn's post hoc test	ns	p = 0.3523	
		NO CORT vs. CORT HIGH	n = 5, 5	Dunn's post hoc test	**	p = 0.0036	
		CORT LOW vs. CORT HIGH	n = 5, 5	Dunn's post hoc test	ns	p = 0.2819	
B	Saccharin preference test		n = 5, 5, 5, 5	Kruskal-Wallis one-way ANOVA		H(3) = 7.768	p = 0.0117
		NO CORT vs. CORT LOW	n = 5, 5	Dunn's post hoc test	ns	p = 0.8639	
		NO CORT vs. CORT HIGH	n = 5, 5	Dunn's post hoc test	*	p = 0.0172	
		CORT LOW vs. CORT HIGH	n = 5, 5	Dunn's post hoc test	ns	p = 0.2673	
C	Duration of immobility in TST		n = 5, 5, 5, 5	One-way ANOVA		F(2, 12) = 2.160	p = 0.1580
Figure 6			**n**	**Statistical test**		**Significance**	
A	Weight changes		n = 11, 9, 9, 10	Kruskal-Wallis one-way ANOVA		H(4) = 11.494	p = 0.009333
		NO CORT VEH vs. CORT VEH	n = 11, 9	Dunn's post hoc test	ns	p = 0.11877	
		NO CORT VEH vs. CORT KET	n = 11, 9	Dunn's post hoc test	*	p = 0.01078	
		NO CORT VEH vs. CORT N2O	n = 11, 10	Dunn's post hoc test	ns	p = 0.09074	
		CORT VEH vs. CORT KET	n = 9, 9	Dunn's post hoc test	ns	p = 1.0	
		CORT VEH vs. CORT N2O	n = 9, 10	Dunn's post hoc test	ns	p = 1.0	
		CORT KET vs. CORT N2O	n = 9, 10	Dunn's post hoc test	ns	p = 1.0	
B	Nest assessment		n = 11, 9, 9, 10	Kruskal-Wallis one-way ANOVA		H(4) = 2.61	p = 0.4551
C	Coat assessment		n = 11, 9, 9, 10	Kruskal-Wallis one-way ANOVA		H(4) = 25.24	p < 0.0001
		NO CORT VEH vs. CORT VEH	n = 11, 9	Dunn's post hoc test	****	p < 0.0001	
		NO CORT VEH vs. CORT KET	n = 11, 9	Dunn's post hoc test	**	p = 0.0017	
		NO CORT VEH vs. CORT N2O	n = 11, 10	Dunn's post hoc test	***	p < 0.0001	
		CORT VEH vs. CORT KET	n = 9, 9	Dunn's post hoc test	ns	p = 1.0	
		CORT VEH vs. CORT N2O	n = 9, 10	Dunn's post hoc test	ns	p = 1.0	
		CORT KET vs. CORT N2O	n = 9, 10	Dunn's post hoc test	ns	p = 1.0	
D	Saccharin preference test		n = 11, 9, 9, 10	One-way ANOVA		F(3, 35) = 8.001	p = 0.000343
		NO CORT VEH vs. CORT VEH	n = 11, 9	Tukey post hoc test	***	p = 0.00035	
		NO CORT VEH vs. CORT KET	n = 11, 9	Tukey post hoc test	ns	p = 0.9102	
		NO CORT VEH vs. CORT N2O	n = 11, 10	Tukey post hoc test	ns	p = 0.8304	
		CORT VEH vs. CORT KET	n = 9, 9	Tukey post hoc test	##	p = 0.00390	
		CORT VEH vs. CORT N2O	n = 9, 10	Tukey post hoc test	##	p = 0.00463	
		CORT KET vs. CORT N2O	n = 9, 10	Tukey post hoc test	ns	p = 0.9985	
E	Duration of immobility in TST		n = 11, 9, 9, 10	One-way ANOVA		F(3, 35) = 3.119	p = 0.0383
		NO CORT VEH vs. CORT VEH	n = 11, 9	Tukey post hoc test	ns	p = 0.2938	
		NO CORT VEH vs. CORT KET	n = 11, 9	Tukey post hoc test	ns	p = 0.3114	
		NO CORT VEH vs. CORT N2O	n = 11, 10	Tukey post hoc test	ns	p = 0.8776	
		CORT VEH vs. CORT KET	n = 9, 9	Tukey post hoc test	ns	p = 0.99998	
		CORT VEH vs. CORT N2O	n = 9, 10	Tukey post hoc test	ns	p = 0.08373	
		CORT KET vs. CORT N2O	n = 9, 10	Tukey post hoc test	ns	p = 0.09042	
F	Latency to immobility in TST		n = 11, 9, 9, 10	One-way ANOVA		F(3, 35) = 2.719	p = 0.0593
		NO CORT VEH vs. CORT VEH	n = 11, 9	Tukey post hoc test	ns	p = 0.3811	
		NO CORT VEH vs. CORT KET	n = 11, 9	Tukey post hoc test	ns	p = 0.0865	
		NO CORT VEH vs. CORT N2O	n = 11, 10	Tukey post hoc test	ns	p = 0.9984	
		CORT VEH vs. CORT KET	n = 9, 9	Tukey post hoc test	ns	p = 0.8586	
		CORT VEH vs. CORT N2O	n = 9, 10	Tukey post hoc test	ns	p = 0.4906	
		CORT KET vs. CORT N2O	n = 9, 10	Tukey post hoc test	ns	p = 0.1327	

Figure 7			**n**	**Statistical test**		**Significance**	
A	*Dusp1*		n = 4, 4, 5, 5	One-way ANOVA		F(3, 14) = 4.260	p = 0.0247
		NO CORT VEH vs. CORT VEH	n = 4, 4	Tukey post hoc test	ns	p = 0.9517	
		NO CORT VEH vs. CORT KET	n = 4, 5	Tukey post hoc test	ns	p > 0.9999	
		NO CORT VEH vs. CORT N2O	n = 4, 5	Tukey post hoc test	ns	p = 0.0883	
		CORT VEH vs. CORT KET	n = 4, 5	Tukey post hoc test	ns	p = 0.9459	
		CORT VEH vs. CORT N2O	n = 4, 5	Tukey post hoc test	#	p = 0.0323	
		CORT KET vs. CORT N2O	n = 5, 5	Tukey post hoc test	ns	p = 0.066	
B	*Dusp5*		n = 4, 4, 5, 5	One-way ANOVA		F(3, 14) = 0.815	p = 0.5065
C	*Dusp6*		n = 4, 4, 5, 5	One-way ANOVA		F(3, 14) = 4.277	p = 0.0244
		NO CORT VEH vs. CORT VEH	n = 4, 4	Tukey post hoc test	ns	p = 0.9742	
		NO CORT VEH vs. CORT KET	n = 4, 5	Tukey post hoc test	ns	p = 0.8112	
		NO CORT VEH vs. CORT N2O	n = 4, 5	Tukey post hoc test	*	p = 0.029	
		CORT VEH vs. CORT KET	n = 4, 5	Tukey post hoc test	ns	p = 0.9694	
		CORT VEH vs. CORT N2O	n = 4, 5	Tukey post hoc test	ns	p = 0.0656	
		CORT KET vs. CORT N2O	n = 5, 5	Tukey post hoc test	ns	p = 0.1124	
Figure 8			**n**	**Statistical test**		**Significance**	
A	*Arc*		n = 4, 4, 5, 5	One-way ANOVA		F(3, 14) = 4.105	p = 0.0277
		NO CORT VEH vs. CORT VEH	n = 4, 4	Tukey post hoc test	ns	p = 0.9174	
		NO CORT VEH vs. CORT KET	n = 4, 5	Tukey post hoc test	ns	p= 0.9175	
		NO CORT VEH vs. CORT N2O	n = 4, 5	Tukey post hoc test	*	p = 0.0321	
		CORT VEH vs. CORT KET	n = 4, 5	Tukey post hoc test	ns	p > 0.9999	
		CORT VEH vs. CORT N2O	n = 4, 5	Tukey post hoc test	ns	p = 0.1086	
		CORT KET vs. CORT N2O	n = 5, 5	Tukey post hoc test	ns	p = 0.0783	
B	*NR4A1*		n = 4, 4, 5, 5	One-way ANOVA		F(3, 14) = 4,904	p = 0.0156
		NO CORT VEH vs. CORT VEH	n = 4, 4	Tukey post hoc test	ns	p = 0.9536	
		NO CORT VEH vs. CORT KET	n = 4, 5	Tukey post hoc test	ns	p = 0.7812	
		NO CORT VEH vs. CORT N2O	n = 4, 5	Tukey post hoc test	*	p = 0.0182	
		CORT VEH vs. CORT KET	n = 4, 5	Tukey post hoc test	ns	p = 0.9773	
		CORT VEH vs. CORT N2O	n = 4, 5	Tukey post hoc test	ns	p = 0.0504	
		CORT KET vs. CORT N2O	n = 5, 5	Tukey post hoc test	ns	p = 0.079	

Appendix 2: Saccharin preference test and tail suspension test results plotted against time. Total immobility time in TST of the CORT N2O group against time of treatment (A) and time between treatment and TST (B). Total immobility time in TST of the CORT KET group against time of treatment (C) and time between treatment and TST (D). SPT results of the CORT N2O (E) and CORT KET (F) group against time between treatment and start of SPT. Data plotted as individual values with a fitted linear curve. TST: tail suspension test, SPT: saccharin preference test.